in the urine, and the kidney must have a considerable power of concentrating formate.

The inhibition of the appearance of formate during the administration of ethanol is in accordance with Zatman's (1946) interpretation of the earlier experiments, namely, that a primary oxidation of methanol to formaldehyde through the agency of alcohol dehydrogenase is competitively inhibited by ethanol. More recently, Jacobsen (1952) has expressed a doubt as to whether alcohol dehydrogenase is involved in methanol oxidation *in vivo*, a doubt based chiefly on the claim of Theorell & Bonnichsen (1951) that pure crystalline alcohol dehydrogenase of horse liver does not react with methanol to any measurable extent. But it has been our experience that cruder preparations of the horse-liver enzyme can easily be shown to reduce diphosphopyridine nucleotide in the presence of methanol; also, for the study of the back-reaction with reduced coenzyme, formaldehyde is in some ways a more convenient substrate to use than is acetaldehyde even with the pure enzyme (Theorell & Chance, 1951). The properties of the pure crystalline enzyme are not necessarily more closely relevant to events *in vivo* than are the properties of the cruder

preparation. Jacobsen's alternative suggestion, that the principal mode of oxidation of methanol *in vivo* may be the catalase-hydrogen peroxide mechanism discovered by Keilin & Hartree (1945), does not at the moment seem to provide a sufficient explanation of the striking inhibitory effect of ethanol. More evidence is required to make it possible to decide whether either of the views so far put forward is correct.

SUMMARY

The appearance of formate in the urine of man, which otherwise follows the administration of a small dose of methanol, was completely inhibited during the repetitive administration of ethanol to the subject. Formate appeared in the urine an hour or two after the cessation of ethanol administration, and its appearance was coincident with the rapid decline in body methanol concentration which then ensues.

It is a pleasure to the authors to express their thanks to Dr A. H. Gowenlock for his ready co-operation as a subject in their experiments.

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A Cholinesterase Inhibitor Derived from *OO*-Diethyl *O-p*-Nitrophenyl Thiophosphate *in vivo*

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(Received 28 October 1952)

The biological action of a highly purified sample of diethyl *p*-nitrophenyl thiophosphate,* hereinafter referred to as parathion, has been studied by Diggle & Gage (1951*a*). They demonstrated that the

* The compounds named here 'thiophosphates' are, according to the nomenclature recently agreed to by the Chemical Society and the American Chemical Society, derivatives of phosphorothionic or phosphorothiolic acids. [Editorial note.]

substance has little or no effect on cholinesterase *in vitro*, although it is a powerful inhibitor of this enzyme *in vivo*. They also succeeded in obtaining inhibition of cholinesterase by parathion *in vitro* in the presence of liver tissue (Diggle & Gage, 1951*b*); the mechanism of this effect has been studied in more detail by Gage & Payton (1952), who came to the conclusion that there exists in liver, and to a lesser extent in other tissues, an enzyme capable of

transforming parathion into a powerful *in vitro* inhibitor of cholinesterase.

A discrepancy between *in vitro* and *in vivo* inhibition of cholinesterase by parathion had previously been noted by other workers, although their figures for the *in vitro* inhibition indicate that their samples of parathion were contaminated with active impurities, and the magnitude of the difference was not fully appreciated. Chamberlain & Hoskins (1951) elaborated a suggestion made by Grob, Garlick & Harvey (1950) that parathion is converted *in vivo* into diethyl *p*-nitrophenyl phosphate, the oxygen analogue of parathion known as paraoxon, which is a potent inhibitor of cholinesterase *in vitro*, although no direct evidence was produced to establish this. Of the substances allied in structure to parathion, and which are known to inhibit cholinesterase, paraoxon appears to be the most likely to be produced by a metabolic change of parathion, particularly as Binkley (1949) has produced evidence to show that there exists in liver an enzyme capable of converting inorganic thiophosphate to phosphate.

This paper reports the extraction of a substance with *in vitro* anticholinesterase activity from the livers of rats dosed with parathion, and a study of its properties in relation to those of paraoxon.

EXPERIMENTAL

Partial purification of erythrocyte cholinesterase. A preparation of cholinesterase was made from horse erythrocytes by a modification of the methods of Mendel & Rudney (1943) and of Mounter & Whittaker (1950).

Washed horse erythrocytes (200 ml.) were diluted to 1 l. with water, 80 g. kieselguhr added and the pH adjusted to 6.3 with 0.1 N-HCl. The suspension was filtered under reduced pressure, the filtrate being returned once through the filter. The residue on the filter was washed with 600 ml. water, and suspended in 100 ml. phosphate saline solution which corresponded to a Krebs-Ringer phosphate buffer (Krebs & Eggleston, 1940) without Ca. The pH was adjusted to 8.0 by addition of 0.1 N-NaOH, and the suspension was then centrifuged. The deposit was suspended again in 100 ml. phosphate saline at pH 8.0 and centrifuged; the two supernatants were bulked together and the pH adjusted to 7.4 with 0.1 N-HCl. Chloroform (ten drops) was added as a preservative and the solution stored in the refrigerator.

Measurement of cholinesterase activity. The activity of the cholinesterase preparation was determined by a modification of the chemical method used by Diggle & Gage (1951a), in which the HCl and FeCl₃ solutions were replaced by 3 ml. acid FeCl₃ reagent prepared by dissolving 10% (w/v) FeCl₃·6H₂O in a mixture of equal volumes of conc. HCl (sp.gr. 1.18) and water. A series of volumes of the cholinesterase preparation was tested in this way and the optical densities corresponding to the unchanged acetylcholine after 15 min. incubation at 37° were measured. These values were subtracted from the optical density corresponding to the initial acetylcholine concentration; these differences were proportional to the amount of acetylcholine hydrolysed

and they were plotted against the volumes of enzyme solution. The volume of enzyme solution which reduced the acetylcholine concentration to one-half was obtained from this curve by interpolation and the enzyme preparation was diluted to contain this volume in 2 ml. This diluted preparation was regarded as having ten arbitrary units of cholinesterase/ml., and the abscissae of the graph were adjusted to read directly in such units; the graph so obtained is shown in Fig. 1.

Fig. 1. Standard curve relating optical density difference between test solution and zero hydrolysis solution with arbitrary units of cholinesterase.

Measurement of inhibitory activity. Into tubes graduated at 15 ml. were pipetted 2 ml. cholinesterase preparation (10 units/ml.) and 1 ml. inhibitor solution. The tubes were incubated for 30 min. at 37° and the residual cholinesterase activity measured as above. By testing various inhibitor concentrations, the concentration required for a 50% inhibition of the cholinesterase was obtained by interpolation.

Preparation of acetone extract of liver from parathion-treated rats. The sample of pure parathion used was the same as that previously examined by Gage (1952). An emulsion of parathion (50 mg./ml.) was prepared by dissolving 100 mg. in 0.2 ml. 50% (w/v) Dispersol OG (Imperial Chemical Industries Ltd.) in ethanol, and making up to 2 ml. with 0.9% NaCl solution. Male rats, mean wt. 230 g., were each injected with 1 ml./kg. body weight of the parathion emulsion into the tail vein under light ether anaesthesia. Death occurred about 10 min. after injection and the liver was immediately removed and dispersed in 3 vol. acetone in a Nelco homogenizer. The suspension was filtered and the residue washed with 1.5 vol. 80% (v/v) aqueous acetone. The bulked filtrates and washings from the livers of twelve dosed rats (132 g. liver) were partially dried over anhydrous Na₂SO₄ overnight. The acetone extract was concentrated under reduced pressure on a water bath at 50°; as the evaporation proceeded solid matter separated and when the liquid commenced to froth it was cooled and filtered with the aid of a little kieselguhr. Evaporation was continued, the filtration being repeated if necessary, until the residual liquid was small in volume and no longer smelt of acetone. The turbid solution was acidified to Congo red with HCl and filtered with the aid of a little kieselguhr, and the filtrate adjusted to about pH 7. After a few hours the solution

could be filtered clear through a Whatman no. 5 paper; it was shown to have a considerable anticholinesterase activity. This aqueous solution was then extracted with two successive portions of 10 ml. benzene, the layers being separated by centrifuging. The anticholinesterase activity of this benzene solution was determined after carefully evaporating a measured volume to dryness and extracting the residue with phosphate-saline; the total activity of the solution was equivalent to that derived from approximately 100 μ g. paraoxon.

Paper chromatography of liver extract

Location of anticholinesterase zone. A strip of Whatman no. 3 MM paper, 35 $\times$ 8 cm., was immersed in a 50% (v/v) solution of propylene glycol in water. The excess solution was removed by blotting paper and the strip was dried at 60° for 2 hr. A spot of the benzene solution of liver extract, equivalent to about 0.7 g. liver, was made at one end of the paper, and level with it a spot of a benzene solution of paraoxon, containing 0.75 μ g. paraoxon. The chromatograms were developed with an 80:20 mixture of petroleum ether (b.p. 60–80°) and benzene, which had been saturated with propylene glycol. When the liquid front had travelled about 25 cm. the paper was removed and air-dried, and cut longitudinally to separate the two chromatograms. Each chromatogram was cut transversely into 2 cm. strips, which were rolled and placed in small test tubes. The anticholinesterase activity of the strips was qualitatively determined by a simplification of the method described above. To each tube was added 4 ml. of the cholinesterase preparation (10 units/ml.) and the tubes were incubated at 37° for 30 min. with occasional shaking. Then 1 ml. 0.004 M-acetylcholine was added and the incubation continued for a further 15 min.; 0.5 ml. of each of the alkaline hydroxylamine and FeCl₃ reagents were added and the contents of the tubes filtered through a small plug of cotton wool. A rough estimation of the degree of inhibition of the cholinesterase was obtained by inspection of the intensity of the brown colour in the filtrate. The results of such an experiment are shown in Table 1.

Location of *p*-nitrophenol derivatives. Chromatograms as described above were prepared using a quantity of the benzene solution of liver extract equivalent to about 7 g. liver and 3 μ g. paraoxon. After the solvent had evaporated, the paper was sprayed with 0.5 N-NaOH and hung in an oven at 60° for several hours. The site of labile derivatives of *p*-nitrophenol could be determined by the yellow spot of sodium nitrophenoxide which appeared on the paper; the rate of development of these spots gave some indication of the ease of hydrolysis of the compound. The chromatogram of the liquid extract showed three spots, one at the origin which appeared immediately on spraying with NaOH due to free *p*-nitrophenol in the extract, a second, with an R_F value of about 0.45, which appeared after heating for less than 1 hr., and a third near the liquid front which took several hours to develop. The position of the middle spot corre-

sponded to the anticholinesterase on the paper in the previous experiment, and was very similar in shape and position to that derived from paraoxon. A similar experiment with parathion alone has suggested that the spot near the liquid front is due to unchanged parathion in the liver extract.

Purification of liver extract

The above experiment indicated that the liver extract contained, in addition to the inhibitory substance, appreciable amounts of *p*-nitrophenol and parathion. The extract was evaporated to dryness and the residue dissolved in about 0.5 ml. *n*-hexane (British Drug Houses spectroscopic grade), and the solution was added to the top of a cellulose powder column (Whatman standard grade) moistened with *n*-hexane. The column was eluted with *n*-hexane and 5 ml. fractions collected. The optical density of the fractions was measured at 265 m μ . in 1 cm. quartz cells by means of a Unicam SP 500 spectrophotometer. Three bands could be separated following closely upon one another. The anticholinesterase activity of the fractions was tested after evaporating 0.1 ml. portions to dryness, and was found to reside in the middle band. The fractions comprising this band were bulked together and evaporated to about 0.5 ml. and submitted to a second chromatographic purification as before. Only one band absorbing at 265 m μ . emerged from the column, and the fractions comprising this band were bulked together. A small portion of this solution was submitted to paper chromatography; only one yellow spot was observed, corresponding in position and shape with that of paraoxon.

Absorption spectrum of inhibitor. The hexane solution of the inhibitor was evaporated to about 5 ml. and its absorption spectrum measured. A single peak at 265 m μ . was observed, similar in shape and position to that of paraoxon. In order to determine the extinction coefficient the hexane solution was evaporated and the residue dissolved in 3 ml. water, and 3 ml. of a 0.05 M-borate buffer, pH 9.85, added. After the absorption spectrum of this aqueous solution had been measured, the solution was used for the next experiment in which the total quantity of *p*-nitrophenol obtained by hydrolysis was measured. From this the extinction coefficients of the solution could be calculated and the absorption spectrum is shown in Fig. 2, together with that obtained from a similar solution of paraoxon (10 μ g./ml.). Both spectra had a peak at 273 m μ .; the extinction coefficient of the inhibitor

Table 1. *Comparison of migration of anticholinesterase of liver extract and of paraoxon on filter paper*

(Number of crosses is an approximate estimation of cholinesterase inhibition.)

Distance from origin (cm.) ...	1	3	5	7	9	11	13	15	17	19	21	23	25
Liver extract	—	—	—	—	—	++	++	+	—	—	—	—	—
Paraoxon	—	—	—	—	—	++	++	+	—	—	—	—	—

was somewhat higher than that of paraoxon, particularly at shorter wavelengths, but it is not impossible that this was due to the presence of an impurity.

Fig. 2. Absorption spectra of inhibitor, $\square$ — $\square$, and paraoxon, $\circ$ — $\circ$, in 0.025 M-borate buffer, pH 9.85.

Fig. 3. Hydrolysis of inhibitor, $\square$, and paraoxon, $\circ$, in 0.025 M-borate buffer, pH 9.85.

Stability of inhibitor. The solution of inhibitor in borate buffer from the previous experiment was maintained at 37° in a stoppered tube, and the absorption spectrum measured at intervals. The peak at 273 mμ. progressively disappeared and was replaced by the peak at 400 mμ. of sodium *p*-nitrophenoxide, from which the extent of hydrolysis could be calculated. When hydrolysis was complete the presence of *p*-nitrophenol in the solution was confirmed and its concentration checked by the colorimetric method of Gage (1952). On the assumption that one molecule of the inhibitor gives rise to one molecule of *p*-nitrophenol, a hydrolysis

curve was constructed; this is shown in Fig. 3, together with a curve for paraoxon derived from an experiment performed under similar conditions. From these figures the hydrolysis constant of both paraoxon and the inhibitor can be calculated to be $2.2 \times 10^{-4} \text{ min.}^{-1}$. From the total amount of combined *p*-nitrophenol present, the concentration of the solution used for the absorption spectrum measurement, and for the subsequent determination of anticholinesterase activity, was shown to be $2.07 \times 10^{-5} \text{ M}$.

Anticholinesterase activity of the inhibitor. A portion of the aqueous solution of the inhibitor used for absorption spectrum measurement was taken for the determination of anticholinesterase activity. The concentration required to produce a 50% inhibition of the enzyme preparation was found to be $3.3 \times 10^{-8} \text{ M}$, which is, within experimental error, the same as that of paraoxon, $2.9 \times 10^{-8} \text{ M}$.

DISCUSSION

Substances other than paraoxon, which are allied in structure to parathion and which are known to inhibit cholinesterase, are the two isomers of parathion, *OS*-diethyl *O*-*p*-nitrophenyl thiophosphate, and *OO*-diethyl *S*-*p*-nitrophenyl thiophosphate. The identity of the former with the inhibitor extracted from the livers of rats dosed with parathion can be disproved by its low stability in alkaline solution, while the latter gives rise to *p*-nitrothiophenol on hydrolysis which does not, on reduction, give the indophenol reaction obtained with reduced *p*-nitrophenol, a reaction which can be obtained from the inhibitor after hydrolysis and reduction.

The evidence from the paper-chromatography experiments indicates that the inhibitor extracted from rat liver is a derivative of *p*-nitrophenol and this is amplified by the observation that the anticholinesterase activity of a purified solution of the inhibitor bears the same relation to the combined *p*-nitrophenol content as obtained with a solution of paraoxon. This observation, together with the similarity between the mobilities of the two substances in a paper chromatogram, their absorption spectra and their rates of hydrolysis in alkaline solution, provides strong evidence that the two substances are identical.

The total amount of the inhibitor extracted from the rat livers is very small, of the order of 0.1% of the parathion injected. The ratio between the amounts of the inhibitor and unchanged parathion from the liver, which can be roughly estimated from the sizes of the two spots on the paper chromatogram, is very much larger. It is probable that this concentration of the inhibitor in the tissues is a balance between its rate of formation from parathion and its rate of destruction by an enzyme of the type shown by Aldridge (1951) to exist in serum.

SUMMARY

1. An acetone extract of the livers of rats dosed with *OO*-diethyl *O*-*p*-nitrophenyl thiophosphate (parathion) has been made, and by paper chromatography the existence of a labile derivative of *p*-nitrophenol which strongly inhibits cholinesterase *in vitro* has been detected.

2. This extract has been purified by chromatography on a cellulose powder column, and the

identity of the inhibitor with diethyl *p*-nitrophenyl phosphate (paraoxon) has been suggested by a comparison of their migration on filter paper, their absorption spectra, their hydrolysis constants and their anticholinesterase activities.

I wish to express my thanks to Messrs Albright and Wilson for a supply of pure parathion, to Wellcome Research Laboratories for a regular supply of horse erythrocytes, and to Miss Sylvia Morrissey for assistance in the experimental work.

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The Assimilation of Amino-acids by Micro-organisms

16. CHANGES IN SODIUM AND POTASSIUM ACCOMPANYING THE ACCUMULATION OF GLUTAMIC ACID OR LYSINE BY BACTERIA AND YEAST

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(Received 31 October 1952)

Many Gram-positive bacteria and yeasts are able to effect a high concentration of certain amino-acids in the free state within the cell (Gale, 1947; Taylor, 1947). In *Streptococcus faecalis* and *Staphylococcus aureus* (*Micrococcus pyogenes* var. *aureus*) lysine enters the cell by diffusion, but the accumulation of glutamic acid only takes place if exergonic metabolism such as glucose fermentation is occurring (Gale, 1947; Najjar & Gale, 1950). In yeasts the accumulation of both lysine and glutamic acid occurs only during the metabolism of glucose (Taylor, 1949). Accumulation of free glutamic acid has been observed in a number of mammalian tissues (Krebs, Eggleston & Hems, 1949) and Terner, Eggleston & Krebs (1950) found that brain cells lose potassium ions when incubated with glucose whereas if glutamic acid is also present, this loss of potassium is prevented. Pulver & Verzá (1940) have shown that potassium leaks from yeast cells during incubation in water and that this leakage is prevented by the fermentation of glucose, but no information is available concerning the changes in sodium or potassium in bacterial or yeast cells during accumulation of amino-acids.

Roberts, Roberts & Cowie (1949), Cowie, Roberts & Roberts (1949) and Roberts & Roberts (1950) have shown that sodium and potassium diffuse readily into the 'water space' of *Escherichia coli* and that, in the presence of glucose, potassium enters into non-ionic combination with breakdown products of glucose and becomes stored within the cell in a non-diffusible form.

Christensen & Riggs (1952) and Christensen, Riggs, Fischer & Palatine (1952*a, b*) have found that mouse ascites carcinoma cells concentrate a number of amino-acids, including the *D*-isomers and 'unnatural' amino-acids such as α -diaminobutyric acid. The accumulation of glycine is accompanied by a loss of potassium from the cells, a gain of sodium and osmotic swelling of the cells. Most of the amino-acids tested displace potassium from the cells, this displacement being almost complete in the case of diaminobutyric acid.

The present investigation was undertaken to determine what changes occur in the sodium and potassium content of bacterial and yeast cells during the accumulation within those cells of glutamic acid or lysine.